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In Situ Modulation of Oxidative Stress: A Novel and Efficient Strategy to Kill Cancer Cells

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Abstract: Cancer cells show an up-regulation of glycolysis, they readily take up vitamin C, and they appear more susceptible to an oxidative stress than the surrounding normal cells. Here we compare, analyse and discuss these particular hallmarks by performing experiments in murine hepatomas (TLT cells) and freshly isolated mouse hepatocytes. The results show that rates of lactate formation are higher in TLT cells as compared to mouse hepatocytes, but their ATP content represents less than 25% of that in normal cells. The uptake of vitamin C is more important in hepatoma cells as compared to normal hepatocytes. This uptake mainly occurs through GLUT1 transporters. Hepatoma cells have less than 10% of antioxidant enzyme activities as compared to normal hepatocytes. This decrease includes not only the major antioxidant enzymes, namely catalase, superoxide dismutase and glutathione peroxidase, but also the GSH content. Moreover, catalase is almost not expressed in hepatoma cells as shown by western blot analysis. We explored therefore a selective exposure of cancer cells to an oxidative stress induced by pro-oxidant mixtures containing pharmacological doses of vitamin C and a redox active compound such as menadione (vitamin K_3). Indeed, the combination of vitamin C (which accumulates in hepatoma cells) and a quinone undergoing a redox cycling (vitamin K_3) leads to an oxidative stress that kills cancer cells in a selective manner. This differential sensitivity between cancer cells and normal cells may have important clinical applications, as it has been observed with other pro-oxidants like Arsenic trioxide, isothiocyanates, Adaphostin.

Keywords: Antioxidant enzymes, ascorbate, glycolysis, hepatoma, menadione redox cycling, vitamin C uptake.

INTRODUCTION

Normal cells respond to external stimuli *via* tightly regulated signalling pathways that either trigger or repress growth. Cancer arises when a cell, for a variety of reasons, escapes the normal brakes placed on its growth and begins to divide in an uncontrolled fashion. This loss of regulation occurs when mutations arise in two broad families of genes that regulate cell growth: oncogenes, which are associated with a dominant gain of function and act as a positive signal for growth; and tumour suppressor genes which are associated with a recessive loss of function. These mutations may be caused by environmental, chemical or biological agents and can result in irreversible alterations in the genome of a cell. However, cancer is relatively rare during an average human lifetime because organisms possess several mechanisms to handle genomic alterations and more than one genetic error is required to generate a tumour. Actually, tumorigenesis appears as a multistep mechanism that reflects the genetic alterations progressively driving a normal tissue to malignancy. The most famous genes whose mutations are frequently associated with the arising of cancers are p53, c-myc, erb B or K ras [1].

Cancer cells are known to present a large genetic heterogeneity. Despite some classical mutations, no typical cancer cell genotype exists and each invasive cancer appears as the consequence of a particular genetic pathway travelled during carcinogenesis [2,3]. In that sense, it is quite surprising to note that the genetic diversity usually presented by cancer cells does not correlate with the clinical observations where a common invasive behaviour, including uncontrolled growth and destruction of normal tissues, is noted. This apparent paradox can be explained in a model of active selection, a phenomenon often described in tumour development as a "Darwinian selection". Indeed, several selective barriers exist within a tumour, namely hypoxia, malnutrition, hormonal fluctuations, numerous attacks by the immune system leading to the selection of adapted cells [4].

Hanahan and Weinberg proposed some years ago, that genetic instability allows a cell to eventually acquire six capabilities that are characteristic of most if not all cancers. These are: self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and the ability to evade apoptosis [5]. Strikingly, these main alterations are shared by most, if not all types of human cancer, but the order in which they are acquired can be variable. Therefore, a complex disease such as cancer becomes understandable in terms of a reduced number of underlying principles. Supporting this hypothesis, it has recently been shown that a simple network of well-defined genetic events is sufficient to convert a healthy cell to a tumorigenic state [6].

Besides these main characteristics, cancer cells also show an almost universal glycolytic phenotype, they accumulate vitamin C, and they have a poor antioxidant status. Therefore, we hypothesized that cancer cell homeostasis may be easily and rapidly impaired as compared to non transformed cells by exposing them to an oxidative stress induced by pro-oxidant mixtures containing pharmacological doses of vitamin C. The rationale is that vitamin C, which is preferentially taken up by cancer cells, reduces redox active compounds such as quinones leading to a futile redox cycling. This redox cycling substantially increases the amount of intracellular reactive oxygen species (ROS) impairing redox homeostasis that cannot be tolerated by malignant cells that are already under high constitutive oxidative stress. Indeed;

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given their lack of antioxidant enzymes, cancer cells are more sensitive towards an oxidative stress than normal cells. In addition, oxidative stress strongly inhibits glycolysis leading to an energetic crisis in cancer cells [7].

The aim of this work is to discuss extensively these hallmarks, namely the acquisition of a glycolytic phenotype, the preferential vitamin C uptake, and the low antioxidant capacities. The analysis of these hallmarks - and their role and influence conditioning a selective cancer cell death - will be illustrated by experiments we performed by using murine hepatomas, namely Transplantable Liver Tumor (TLT) cells, and freshly isolated mouse hepatocytes. At the end, the potential clinical consequences of this differential sensitivity between cancer cells and normal cells will be discussed.

PARTICULAR CANCER CELL FEATURES MAKING THEM POTENTIAL TARGETS FOR AN OXIDANT INJURY

1. Glycolytic Phenotype Acquisition

Among the classical features presented by cancer, the up-regulation of glycolysis is probably one of the earliest described, since the first observations were made 80 years ago by Warburg [8]. It is well known that glycolytic rates increase in neoplasms, often proportionally to the degree of the malignancy [9]. Nevertheless, this nearly universal phenotype has never been fully investigated, with the exception of these last years with the widespread clinical use of ^{18}fluorodeoxyglucose positron-emission tomography (PET-SCAN). PET imaging has demonstrated that the glycolytic phenotype is observed in most human cancers and that the rate of glucose consumption can be directly related to tumour aggressiveness (and therefore prognosis). Moreover, this high rate of glucose consumption explains why a hypoglycemic effect may arise in patients bearing large tumours [10].

Several hypotheses have been proposed to explain the persistent metabolism of glucose to lactate even under aerobic conditions, the so-called "Warburg's effect". Among them, the most frequently evoked are: a decrease in the pO_2 (hypoxia), the overexpression of some mitochondrial enzymes and/or the occurrence of some mitochondrial defects.

a. Role of Hypoxia

The acquisition of the glycolytic phenotype by cancer cells due to a reduction in the normal level of oxygen tension may be explained by different mechanisms.

- The uncontrolled proliferation of cancer cells leads to the colonization of areas at increasing distance from blood vessels. Due to the poor oxygen diffusion, this rapidly generates a gradient of oxygen within the growing tumour [11]. This fact, coupled to the increasing metabolic demands of the growing mass of cells provokes a chronic hypoxia, even in tumours of only a few cubic millimetres.
- Due to the particular features in the tumour microenvironment (low pH, low pO_2, high vascular permeability,...), coupled to the tortuous tumour vasculature, a very unstable blood flow is observed that provokes the appearance of areas of fluctuant hypoxia in tumours [12].
- Hypoxia-reoxygenation cycles generate free radicals that can damage the tumour genome, leading to a higher mutation frequency in tumours and the putative selection of mutants possessing a growth advantage. Therefore, hypoxic areas within tumours are the places where cells are the most aggressive, due to both radioresistance and increase in the environmental selective pressure because of nutrient deprivation [13].
- The adaptation of cancer cells to hypoxia notably arises from the activation of transcription factors such as HIF-1 [14]. HIF-1 is a heterodimer that consists of a constitutively expressed HIF-1β subunit and a tightly regulated HIF-1α subunit. The expression of this latter is controlled by the levels of O_2: under normoxic conditions, HIF-1α is degraded by the 26S proteasome after hydroxylation of proline residues in HIF-1α by proline hydroxylases. Under hypoxic conditions, the activity of prolyl hydroxylases decreases leading to the transcriptional activation of HIF-1 target genes like angiogenic factors, glycolytic enzymes, survival factors and invasion factors. Therefore, the activation of HIF-1 provides an explanation for the high levels of some key glycolytic proteins found in cancer, such as glucose transporters (GLUTs) [15,16].

b. Overexpression of Mitochondrial Enzymes

The overexpression of the mitochondrial-bound isoforms of hexokinase (HK-I and HK-II) is another hypothesis explaining the glycolytic phenotype exhibited by cancer cells [17]. Such an up-regulation occurs by epigenetic changes (hypomethylation) that allow an open conformation of the promoter, thus enhancing the binding of transcription factors. Since the mitochondrial-bound isoforms of hexokinase have an easier access to ATP, they are less susceptible to inhibition by their product (glucose-6-phosphate) and present a low Km for glucose. Therefore, they act as a trap mechanism for the capture of glucose and greatly increase the rate of aerobic glycolysis [10].

c. Mitochondrial Defects and Impairment of Oxidative Phosphorylation

The acquisition of the glycolytic phenotype has been proposed to be the consequence of mitochondrial defects, leading to the impairment of oxidative phosphorylation. While the occurrence of a respiratory defect in cancer cells is still extensively debated, significantly reduced levels of β-F_1-ATPase have been observed in several types of cancer, linked to an altered bioenergetic mitochondrial phenotype [18]. One hypothesis is that the mitochondrial DNA, lacking histones and relative protective systems, is much more sensitive to mutations than nuclear DNA. Moreover, since the whole mitochondrial genome is required to maintain mitochondrial functions (absence of introns), only a small change in mitochondrial DNA leads to deleterious effects on the electron transport [19]. However, the great number of mitochondria per cell (200-2000) as well as the presence of several genomes per mitochondrion (2-10 genomes) indicates that the mitochondrial genome can support up to 90 % of

damaged DNA through complementation by the wild-type [20]. In that sense, the precise mechanism underlying the alteration of mitochondrial function in cancer remains elusive and the question is still open whether this phenomenon is a causative link to the process of cancer or simply a secondary bystander effect.

Although the up-regulation of glycolysis is usually considered the consequence of cancer cell adaptation to extreme microenvironmental conditions, its role in invasiveness is now suspected [21,22]. Recently, it has been hypothesized that the increased consumption of glucose in metastatic lesions is not used for substantial energy production *via* Embden-Meyerhoff glycolysis, but rather for production of acid, which gives the cancer cells a competitive advantage for invasion [23-25]. Then, cancer cells can progress following the peritumoral acid gradient and progressively replace the surrounding healthy tissue where cells are dead. We decided then to compare the formation of lactate in both murine hepatomas (TLT cells) and freshly isolated mouse hepatocytes. Cells were incubated in the presence of glucose and lactate formation was recorded during 3 hours according to procedures reported elsewhere [7]. In agreement with a previous report [26], we observed an enhanced rate of about 9-fold in the lactate formation in cancer cells as compared to healthy cells (Fig. **1a**).

Given that the acquisition of a glycolytic phenotype represents a key event for both survival and progression of cancer, the inhibition of glycolysis represents then a novel promising target for cancer therapy [27,28]. Moreover, since an impairment of oxidative phosphorylation has been observed in several types of cancer [18], the cellular ability to produce ATP should be reduced in cancer cells as compared to healthy cells. Indeed, it should be noted that lower ATP levels have been reported in cancer cells as compared to healthy cells [26]. Accordingly, we observed that the intracellular content of ATP in hepatoma cells represents less than 25% of that in normal hepatocytes (Fig. **1b**).

Our results clearly show that cancer cells are producing more lactate than normal cells, indicating that they have a glycolytic phentotype. In addition, they synthesize less nanomoles of ATP per mg of proteins as compared to healthy cells. On the basis of these previous data, it is reasonable to hypothesize that by targeting a critical pathway like glycolysis, it should be expected to obtain a more important cancer cell death.

2. Accumulation of Vitamin C

Humans lack the gulonolactone oxidase enzyme, one of the key enzymes in the biosynthesis of ascorbic acid, and therefore, they should find their high requirements in foods, notably in fruits and vegetables [29]. Ascorbic acid is readily absorbed from the intestine and the absorption of dietary ascorbate is nearly complete. Following its absorption, ascorbic acid is ubiquitously distributed in the cells of the body. Within the body, the highest levels of ascorbic acid are found in the pituitary gland, the adrenal glands, the various white blood cells and the brain.

Actually, the transport of ascorbate and its oxidized form, dehydroascorbic acid (DHA), is mediated by different systems. In specialized cells, vitamin C is directly transported as ascorbic acid by sodium-dependent transporters SVCT1 and SVCT2 [30-32]. While SVCT2 is widely expressed, SVCT1 is largely confined to the bulk transporting epithelial systems (intestine, kidney, liver) and other epithelial tissues (lung, epididymus and lacrymal gland). However, most cells transport vitamin C as DHA, *via* facilitative glucose transporters (Glut), including Glut1 [33]. Glut1 is ubiquitously expressed in cells and up-regulated by malignant transformation [34]. Since DHA is rapidly reduced on the internal side of the plasma membrane, this prevents its efflux and allows the accumulation of ascorbate against a concentration gradient [29,35].

The question of a preferential accumulation of vitamin C in tumours as compared to healthy tissue still remains a sub-

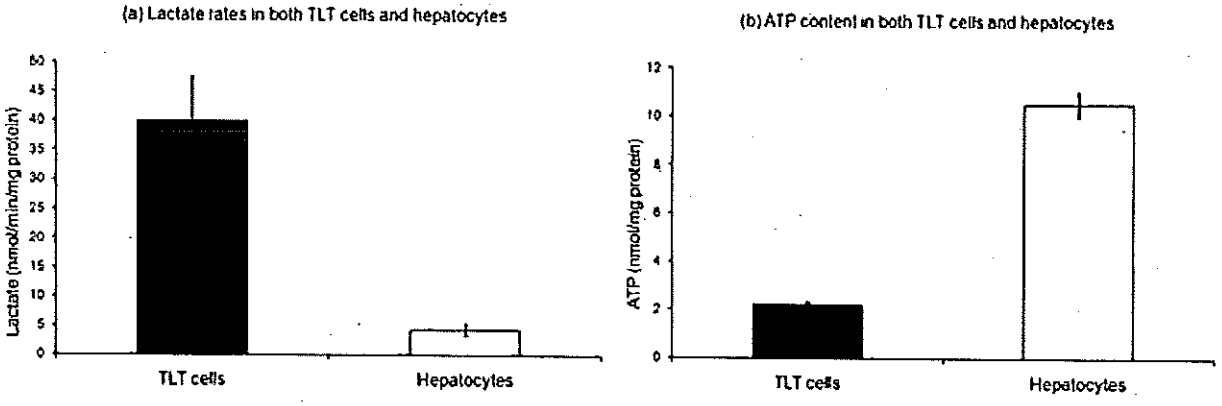

Fig. (1). Formation of lactate and ATP content in both hepatomas and hepatocytes.

(a) Cells were incubated during 120 min in Krebs-Henseleit medium containing 10 mM glucose, and rates of lactate formation were determined by measuring its conversion into pyruvate hydrazone while NAD is converted into NADH as reported elsewhere [7]. The results are expressed as nmol of lactate formed/min/mg of protein. (b) The intracellular ATP content was measured on neutralized extracts using the ATP Bioluminescence Assay Kit CLS II from Boehringer-Mannheim (Germany). The results are expressed as nmol ATP/mg protein. The amount of protein was determined by the Lowry method [77], using bovine serum albumin as standard.

ject of controversy [36]. Indeed, by comparing neoplastic and non-neoplastic breast tissue samples from the same patient, it was found that ascorbic acid was greatly increased in the epithelium of neoplastic tissue as compared to the adjacent tumour-free tissue [37]. In another study, however, significantly increased levels of ascorbic acid were observed in tumour tissue and in tumor-free tissue of oral squamous cell carcinoma patients as compared with healthy subjects. Interestingly, a decrease in ascorbic acid was observed in the blood of oral cancer patients, as compared with healthy subjects [38]. It should be noted that this low concentration of ascorbic acid in plasma was the basis for Pauling's proposals about the use of vitamin C in cancer therapy. Recently, low levels of vitamin C in cancer patients have been reported by Gonçalvez *et al.* [39], who showed that plasma vitamin C was lower in the cervical carcinoma group when compared to control.

Since Glut1 is up-regulated by malignant transformation [34], we further examined if cancer cells take up vitamin C more than healthy cells. To this end, we performed *in vitro* incubations of both murine hepatoma cells (TLT cells) and freshly isolated mouse hepatocytes in the presence of different concentrations of vitamin C ranging from 0 to 4 mM. It should be noted that this latter concentration corresponds to pharmacological doses of ascorbate that may be easily reached by intraperitoneal or intravenous injection [40,41]. In addition, to check the major role of the glucose transporter Glut1, experiments were done in the absence and in the presence of 2-deoxyglucose, an inhibitor of this transporter [42]. In agreement with previous reports showing that cancer cells readily take up vitamin C *in vitro* [43,44], our results clearly show that the uptake of vitamin C is more important in TLT cells as compared to normal hepatocytes (Fig. **2a**). In addition, our results also show that the vitamin C uptake is strongly reduced in the presence of 2-deoxyglucose (Fig. **2b**), indicating that Glut1 plays a major role in this uptake.

Thus, our experimental results tend to confirm one important feature of cancer cells, namely, the rapid and preferential uptake of vitamin C. Later on, we will discuss the significance of this preferential accumulation.

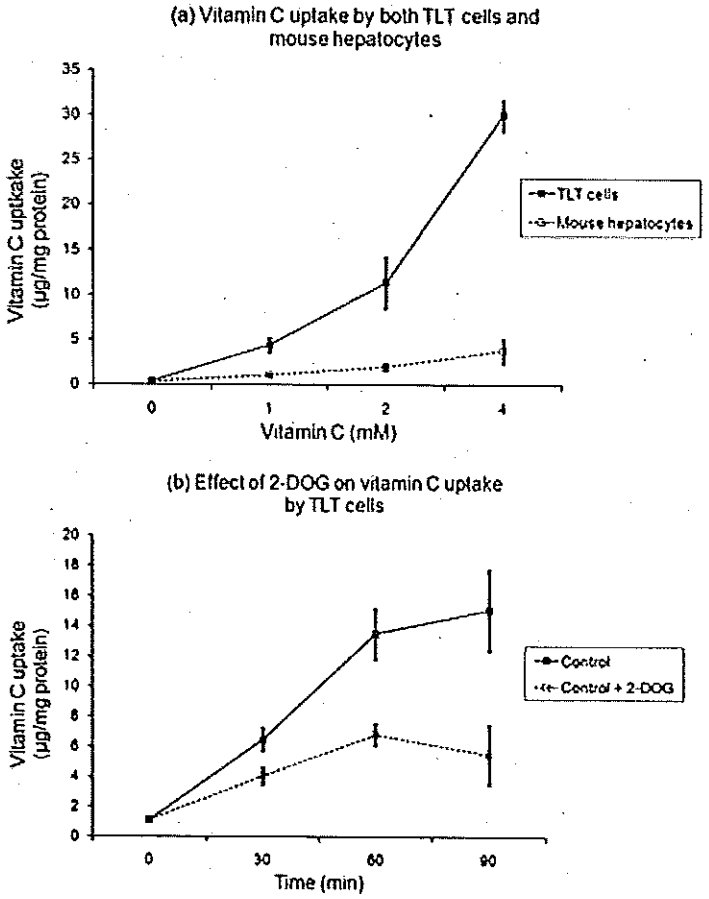

Fig. (2). Intracellular accumulation of vitamin C uptake in both hepatomas and hepatocytes.

(a) Cells were incubated for 60 min in the presence of different concentrations of vitamin C, and its cellular uptake was determined by HPLC as reported elsewhere [41]. Briefly, samples were deproteinized by using metaphosphoric acid, and supernatants, collected after centrifugation, were immediately processed. Separations were achieved by using a Nucleosil C18 column. Detection was set at 254 nm and the flow rate was 1 mL/min. Results are expressed as µg vitamin C/mg protein. (b) TLT cells were preincubated for 60 min in the absence or in the presence of 30 mM of 2-Deoxyglucose (2-DOG). Thereafter, cells were incubated for 90 min in the presence of 2 mM of vitamin C.

3. Low Antioxidant Capacities

It is known that overproduction of reactive oxygen species (ROS) is involved in the initiation and progression of cancers, DNA damage, genetic instability, cellular injury, alterations in drug sensitivity and cell death [45,46]. Indeed, at the beginning of the cancer process, oxidative conditions are often associated with carcinogenicity [47]. During its progression, some oxygen species like hydrogen peroxide can mediate signal transduction and promote cell proliferation [48]. On the other hand, tumour cells appear more susceptible to an oxidative stress than normal cells most probably by a differential redox control of proliferation and viability in non-transformed versus malignant cells [49]. In addition, due to the increased metabolic stress and proliferative capacity of cancer cells, the constitutively high levels of cellular oxidative stress and dependence on ROS signalling may represent a redox vulnerability of malignancy that can be targeted by chemotherapeutic intervention using redox modulators [50,51].

Although it may be hazardous to make a global conclusion about a poor antioxidant status in cancer cells, several studies have reported an imbalance in antioxidant enzyme levels in cancers compared with the cell of origin. For instance, the activities of copper- and zinc-containing superoxide dismutase (CuZnSOD), catalase and glutathione peroxidase appear to be decreased in tumours [52-58]. Furthermore, due to a constant adaptation of cancer cells to their environment, the loss of antioxidant enzymes could be considered as a normal process in cancer progression like in oral squamous cell carcinoma [59]. The situation is more complex with manganese superoxide dismutase (MnSOD) since its expression appears to be either decreased [60], or increased in human tumours [61]. In that sense, the recently discovered thioredoxin (Trx) system perfectly reflects this ambiguity. Indeed, it appears that many human tumours exhibit an overexpression of thioredoxins, possibly linked to a resistance to chemotherapy [62]. These high levels of thioredoxins were notably found to correlate positively with cell proliferation and negatively with apoptosis. However, since thioredoxins have been shown to possess various cellular activities including growth-stimulating properties, it is difficult to evaluate whether these features are linked or not with their antioxidant capacity.

Results obtained in our laboratory show that in cancer cells, the antioxidant enzyme activities represent less than 10% as compared to normal hepatocytes. Such a decrease includes major antioxidant enzymes, namely catalase, super-

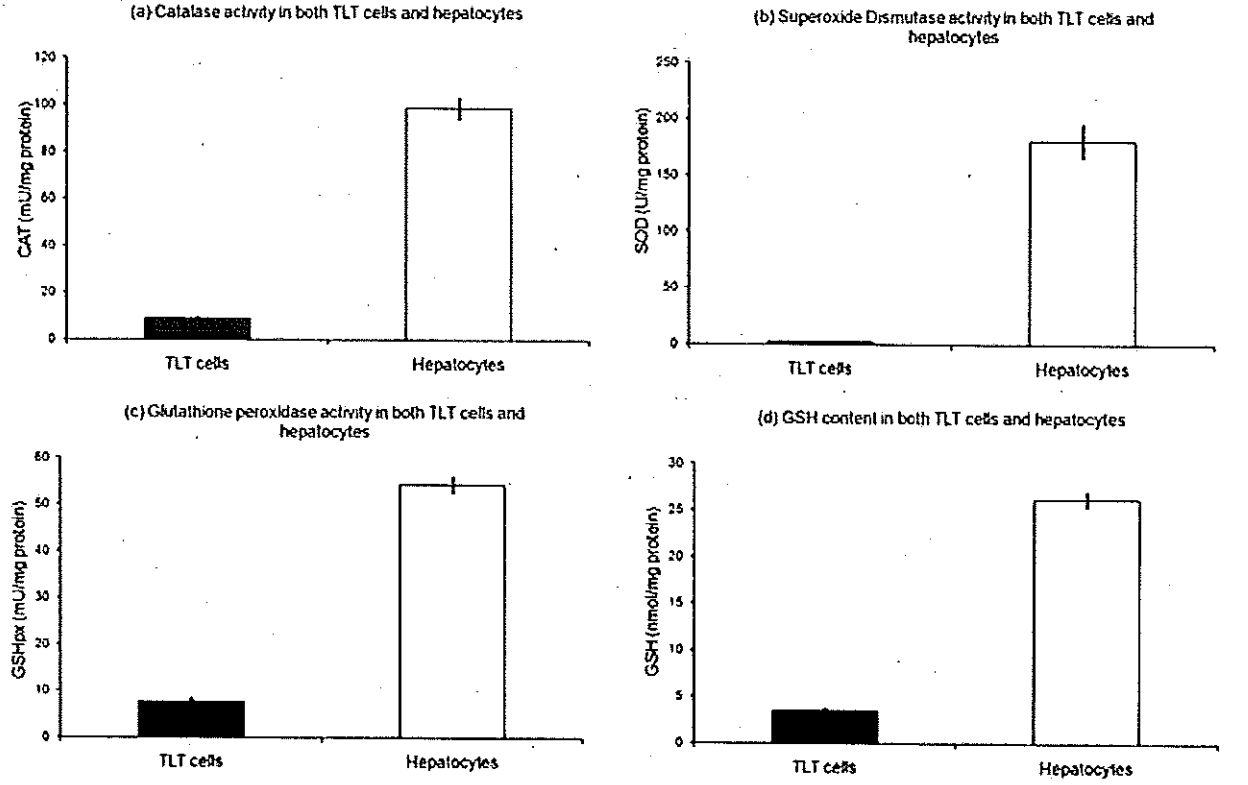

Fig. (3). Antioxidant enzyme activities and GSH content in both hepatomas and hepatocytes.

The enzymatic activities were measured following the procedures reported elsewhere [58]. (a) Catalase activity was measured by using the $TiSO_4$ method and the results are expressed as mUnits/mg protein. (b) the activity of SOD was measured by recording the reduction of nitro blue tetrazolium and the results are expressed as Units/mg protein. (c) The activity of GSHpx was determined by following the oxidation of NADPH and the results are expressed as mUnits/mg protein. (d) The intracellular content of GSH was determined in clean-supernatants using a modified method of Hissin and Hilf [78], after the formation of a fluorescent complex with o-phthalaldehyde (oPT) and measurement at 345 nm excitation and 420 nm emission. The results are expressed as nmol GSH/mg protein.

oxide dismutase, glutathione peroxidase as well as the intracellular content of GSH (Fig. **3a-d**).

Under our experimental conditions, we were able to show that the low activities of the enzyme catalase (CAT) are probably explained by the low amount of proteins since the enzyme was almost not expressed in TLT cells as compared to normal mouse hepatocytes as shown by western blot analysis (Fig. **4**).

4. Effects on Hepatocarcinoma Survival by Using Pharmacological Doses of Ascorbate and a Redox Active Quinone

By targeting three features of cancer cells, namely acquisition of a glycolytic phenotype, a preferential vitamin C uptake and a poor antioxidant status, we developed a strategy to kill cancer cells in a selective way. Indeed, the rationale of our approach is that an oxidative stress induced by a pro-oxidant ascorbate activity may impair glycolysis. The impairment of this critical pathway provokes an energy failure rendering cancer cells particularly sensitive to chemical mixtures containing pharmacological doses of ascorbate and redox active compounds. This explanation is likely underlying the *in vivo* antitumour effect shown by the combination of ascorbate and menadione as reported by Taper and colleagues during the last 20 years. Briefly, in TLT bearing mice, Taper *et al.* has shown a potentiating effect of both chemo- and radiotherapy [63,64]; a sensitisation of cancer chemotherapy resistant to Vincristin [65]; and finally, an inhibition of metastases development [66].

Since this potential anticancer effect may be biologically relevant, *in vitro* experiments were performed to explore if these cancer features are involved in the mechanisms by which ascorbate/menadione is killing TLT cells. Thus, we have previously shown that the incubation of TLT cells in the presence of pharmacological doses of ascorbate (2 mM) and a redox active quinone (menadione) leads to a necrosis-like cell death. Such a cell death is most probably induced by H_2O_2 generated by vitamin C-driven menadione-redox cycling; it is preceded by DNA strand breaks that do not correspond to oligosomal DNA fragmentation (a hallmark of apoptosis), and by inhibition of NF-kB constitutive activity; and do not correlate with activation of MAP kinases [58,67]. To go further, we analysed the role of the reducing agent as well as of the redox active compound. To this end, TLT cells were incubated in the absence and in the presence of ascorbate/menadione during 4 hours. The combination of vitamin C (which accumulates in TLT cells) and a quinone undergoing a redox cycling (menadione or vitamin K_3) leads to an oxidative stress that rapidly kills cancer cells (Fig. **5a**). Such a cell death was completely blocked by adding the antioxidant N-acetylcysteine (NAC), while the preincubation of TLT cells with 3-aminotriazole (ATA), still enhances the LDH leakage (Fig. **5b**). When vitamin E was employed as reducing agent instead of vitamin C, its combination with 50 µM menadione (vitamin K_3) did not lead to cytotoxicity (Fig. **5c**). Indeed, the LDH leakage observed in cells incubated with menadione (K_3) alone was about 30%, a similar value to that observed with the combination vitamin E/menadione (E/K_3). Meanwhile, the association ascorbate/menadione (CK_3) provokes more than 90% of LDH leakage. Finally, it should be underlined that cell death was only observed when the quinone derivative has a redox potential high enough to oxidize ascorbate thus initiating a futile redox cycling (Fig. **5d**). Indeed, the mixtures of vitamin C with menadione (CK_3) or naphtoquinone (C/NQ) were able to kill cells, while mixtures containing vitamin K_1 (CK_1) did not induce cell death. These results are in agreement with previous data we obtained by using other quinones and testing the cytotoxic effect in K562 cells, a human leukaemia cell line [67].

In addition, after 2 hours of incubation in the presence of ascorbate/menadione, rates of lactate formation were decreased by 80% while the ATP content was decreased by 90% as compared to untreated TLT cells (data not shown). These data are in agreement with previous results obtained with human cancer cell lines [7]. Based on all these previous features, we can conclude that vitamin C (at pharmacological doses) reduces a redox-active quinone such as menadione generating a redox cycling that results in a large, and sustained, amount of ROS. This causes an oxidative stress that impairs glycolysis, depletes ATP cellular contents and kills cancer cells.

CONCLUDING REMARKS

Three major conclusions arise from the results reported here as well as from the several studies conducted in our laboratory during the last twenty years. First, ascorbate by

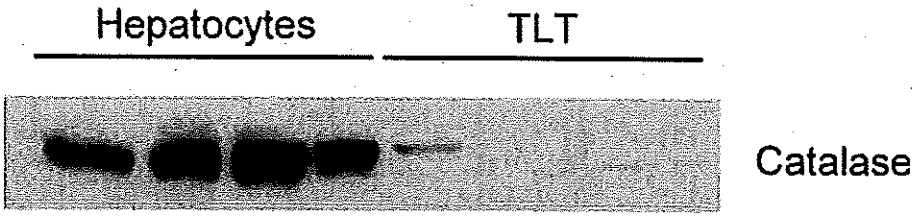

Fig. (4). Catalase expression in both hepatomas and hepatocytes.

Cells were washed twice with ice-cold PBS and then resuspended in a lysis RIPA buffer supplemented with one tablet of Complete Mini protease inhibitor cocktail. The samples were kept on ice for 20 min, centrifuged at 13,000 × g for 20 min at 4°C. Supernatants were collected and then stored at −80°C. Equal amounts of proteins were subjected to SDS-PAGE (6–15% separating gel) followed by electroblot to nitrocellulose membranes. The membranes were blocked 1h in TBS buffer (pH 7.4) containing 5% powdered milk protein and then incubated overnight at 4°C with rabbit polyclonal antibodies against catalase (Cell Signaling Technology, Danvers, MA). After washing, membranes were exposed during 60 minutes at room temperature to a secondary antibody from Chemicon International (Temecula, CA) linked to HRP. Finally the protein bands were detected by chemiluminescence using ECL detection kit (Amersham, UK).

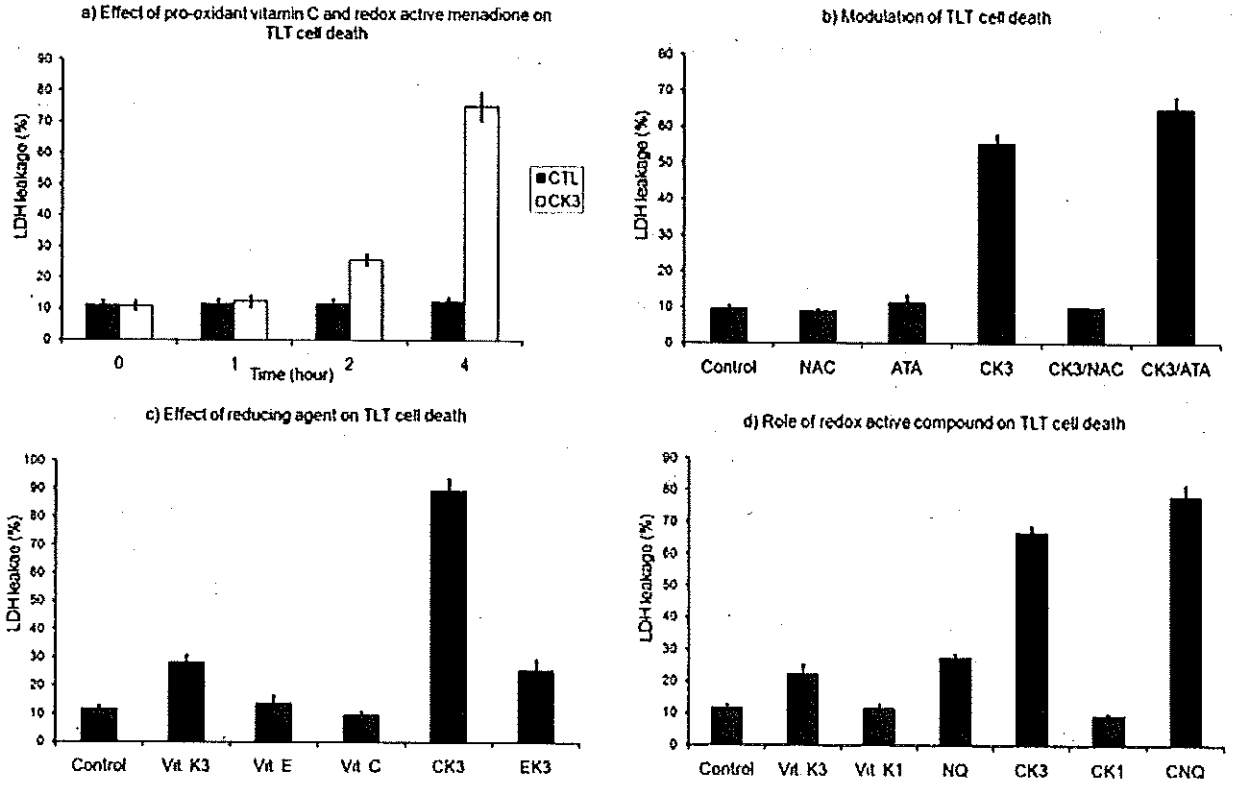

Fig. (5). Role of reducing agents and redox active compounds on TLT cell survival.

Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH), according to the procedure of Wrobleski and Ladue [79], both in the culture medium and in the cell pellet obtained after centrifugation. The results are expressed as a ratio of released activity to the total activity. Briefly, TLT cells were incubated for 4 hours in the absence (control) and in the presence of different compounds, either alone or combined as indicated in the respective figures. (**a**) vitamin K_3 (10 μM) and vitamin C (2 mM). (**b**) vitamin K_3 (10 μM), vitamin C (2 mM), 3 mM of N-acetylcysteine (NAC); and 5 mM of 3-aminotriazole (ATA). (**c**) vitamin E (2 mM), vitamin C (2 mM), vitamin K_3 (50 μM). (**d**) vitamin K_1 (50 μM), napthoquinone (50 μM), vitamin C (2 mM), and vitamin K_3 (50 μM).

reducing redox active quinones leads to the formation of ROS, particularly hydrogen peroxide (H_2O_2) which generates a mild but sustained oxidative stress killing cancer cells in a selective way. As outlined in Fig. (6), vitamin C is taken up by cells either as ascorbate (AsH) *via* the SVTC transporters or as dehydroascorbate (DHA) *via* the GLUT1 transporters. Since these latter are overexpressed in cancer cells, a higher accumulation of vitamin C occurs in hepatomas as compared to hepatocytes. Within the cells, DHA is reduced to AsH which then reduces the quinone (Q) into the semiquinone ($SQ^{\cdot}$). In the presence of oxygen, $SQ^{\cdot}$ is oxidized back to Q, and O_2 is reduced to superoxide anion ($O_2^{\cdot-}$). By spontaneous or catalysed dismutation, $O_2^{\cdot-}$ leads to hydrogen peroxide (H_2O_2). Since in hepatomas there is less catalase (CAT) and glutathione peroxydase (GSHpx) to detoxify H_2O_2 than in hepatocytes, this oxidizing species generates an oxidative stress which is lethal for cancer cells.

Second, the potentiation of chemotherapy reported by Taper and coworkers in TLT-bearing mice suggests a non-specific process which is not depending on the chemotherapeutic agents. Moreover, ascorbate and menadione, when used in combination, exhibit a synergistic action and are devoid of toxicity. Finally, given the strong dependence on glycolysis shown by cancer cells, their intracellular accumulation of vitamin C and their poor antioxidant status, the third conclusion (and maybe the most important) is that the ascorbate/menadione appeared to be selective for cancer cells. This "apparent selectivity", however, is more a "differential sensitivity" between healthy and cancer cells, as previously described by Zhang *et al.* [68]. It should be noted that the action of these compounds is not related to their vitamin action, but rather involves a cytolytic process that takes advantage of tumour metabolism. We postulate that the consequences of this differential sensitivity between cancer cells and normal cells may have important clinical applications.

Indeed, despite the constant efforts furnished in the fight against cancer and the progress achieved in medicine, cancer is still a leading life-threatening pathology and its impact and cost to our society is still huge. For the year 2005, according to the National Cancer Institute, the number of new cases and deaths for the US were expected to be more than 1 370 000 and 570 000, respectively. With an estimated 3 190 000 new cases and 1 700 000 deaths for 2006, cancer remains also an important public health problem in Europe [69]. Classical treatments of cancer consist in surgical removal, radiotherapy and chemotherapy. Since many years, other treatment methods are under development, and in particular angiogenesis inhibitors and immunological therapies (mono-

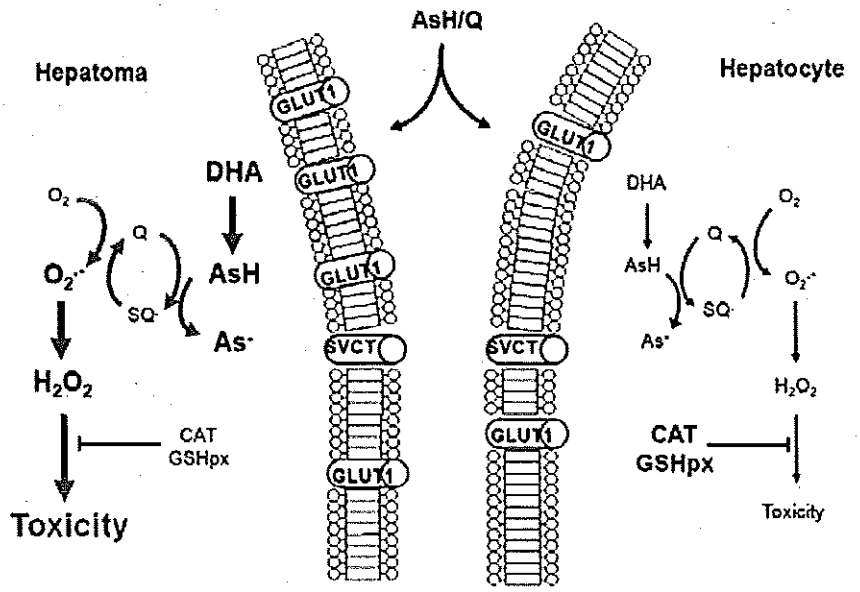

Fig. (6). Vitamin C uptake and further quinone redox cycling leading to cancer cell death.

Vitamin C is taken up by cells mostly as dehydroascorbate (DHA). Within cells, DHA is reduced back to ascorbate (AsH), which initiates a redox cycle by electron transfer from AsH to menadione (Q) leading to the formation of semiquinone radical (SQ·). The rapid reoxidation of SQ· to its quinone form (Q) leads to an enhanced oxygen uptake, and the generation of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2).

clonal antibodies, cancer vaccine, etc) seem to be highly promising. Nevertheless, many clinical trials are still required in order to evaluate their effectiveness as well as their safety. Most of these new therapies arise from our better knowledge of cancer biology, e.g. imatinib (Gleevec®) that targets the chimeric protein Bcr-ABL whose constitutive tyrosine-kinase activity is responsible for the tumorigenicity of certain leukaemic cells. However, mutations within the kinase domain of Abl may interfere with the binding of the drug, leading to drug resistance. This perfectly illustrates the great importance of our understanding of the biology of cancer in order to develop future therapeutics. Accordingly, since ROS have been associated with tumour formation either as a causative effect or as a result of oncogenic transformation, and due to a low antioxidant capacity of cancer cells, such a precarious redox equilibrium renders them highly sensitive to a further oxidative stress [70,71]. For instance, the up-regulation of the PI3K/AKT pathway, one of the most prevalent alterations in human cancer, leads to ROS sensitization due to FoxO inhibition and subsequent repression of both MnSOD and catalase expression [72]. Such a redox phenotype is conditioning the response of cancer cells to several pro-oxidant agents which, by modulating ROS formation, have been shown to induce cancer cell death. Among pro-oxidant drugs, Adasphostin, an adamantyl ester of AG957, initiates apoptosis in human leukemia cells in association with generation of ROS [73]. Moreover, arsenic trioxide, used as anticancer agent in traditional Chinese medicine, also induces apoptosis in cancer cells by a hydrogen peroxide-mediated process [74,75]. Finally, β-phenylethyl isothiocyanate (PEITC), a natural compound found in vegetables, has been shown to induce oxidative stress and a subsequent cell death in chronic myeloid leukaemia cells [50,76]. These few examples show that it is possible to take advantage of this cellular redox imbalance and to modulate intratumoral ROS formation to selectively kill cancer cells.

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